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The origin of the high sensitivity of muscle fructose 1,6-bisphosphatase towards AMP

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Abstract **Adenosine 5′-monophosphate (AMP) inhibits muscle fructose 1,6-bisphosphatase (FBPase) about 44 times stronger than the liver isozyme. The key role in strong AMP binding to muscle isozyme play K20, T177 and Q179. Muscle FBPase which has been mutated towards the liver enzyme (K20E/T177M/Q179C) is inhibited by AMP about 26 times weaker than the wild-type muscle enzyme, but it binds the fluorescent AMP analogue, 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-monophosphate (TNP-AMP), similarly to the wild-type liver enzyme. The reverse mutation of liver FBPase towards the muscle isozyme significantly increases the affinity of the mutant to TNP-AMP. High affinity to the inhibitor but low sensitivity to AMP of the liver triple mutant suggest differences between the isozymes in the mechanism of allosteric signal transmission.**

Keywords: Muscle FBPase; Allosteric inhibition; AMP binding; Glyconeogenesis

1. Introduction

Fructose 1,6-bisphosphatase (FBPase, EC 3.1.3.11) catalyzes hydrolysis of fructose 1,6-bisphosphate (F1,6-P_2) to fructose 6-phosphate (F6-P) and inorganic phosphate in the presence of such divalent metal ions as: Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} [1,2]. Contrary to previously mentioned divalent cations, Ca^{2+} inhibits FBPase activity [3,4]. Both mammalian FBPase isozymes – liver and muscle – are activated by monovalent cations [1,2,5,6] and are inhibited competitively by fructose 2,6-bisphosphate (F2,6-P_2) and allosterically by Adenosine 5′-monophosphate (AMP) [1,2,5–8].

Mammalian FBPases are homotetrameric enzymes with a subunit molecular weight of about 37 kDa [9–12]. The tertiary structure of each monomer is composed of two domains, the F1,6-P_2 domain containing the active site and the AMP domain with the AMP binding site.

The liver FBPase is recognized as regulatory enzyme of gluconeogenesis. Proposed functions of the muscle isozyme are participation in glycogen synthesis from lactate [13,14] and regulation of glycolysis via futile cycle which may be formed with phosphofructokinase (PFK) [15]. The basic difference in the kinetic properties of the liver and muscle isozymes concerns their sensitivity to calcium ions [3,4] and to AMP inhibition [2,16–18]. In case of the muscle enzyme $I_{0.5}$ for AMP is about 0.1 μM, i.e., 50–100 times lower than the corresponding value determined for liver FBPase. The high sensitivity of muscle FBPase indicates that the isozyme should be almost completely inhibited in vivo [19–21].

Recently, we have shown that interaction of muscle FBPase with muscle aldolase in vitro results in the formation of a complex in which FBPase is entirely insensitive to AMP inhibition [20,22] and strongly desensitized to Ca^{2+} inhibition [4] and F1,6-P_2 is transferred directly from aldolase to FBPase [22]. It is presumed that sarcomere-bound FBPase interacts with aldolase and other glyconeogenic enzymes forming a multienzyme complex within the region of the Z-line which synthesizes glycogen from carbohydrate precursors [4,22–24]. On the other hand, the fraction of uncomplexed FBPase is supposed to increase during acceleration of glycolysis [20]. Thus, the high sensitivity of free FBPase to AMP inhibition seems to be a mechanism preventing muscle cells from dissipating energy via futile cycling between PFK and FBPase during muscle contraction [4,25].

The molecular basis of high sensitivity of muscle FBPase to AMP inhibition is unknown. Recently, we hypothesized that the key role in stronger AMP binding to the muscle isozyme is played by K20, T177 and Q179 which allow the formation of new hydrogen bonds to the inhibitor [21]. In the present report, we provide experimental data demonstrating the crucial role of residues 20, 177 and 179 for a different binding of AMP to liver and muscle FBPase. Our results also suggest that an additional mechanism connected with allosteric signal transmission in muscle FBPase must account for the strong inhibition of the muscle enzyme.

2. Materials and methods

Phosphocellulose P-11 was purchased from Whatman (Maidstone, England), ammonium sulfate and Coomassie Brilliant Blue were from Fluka (Buchs, Switzerland). 2′-(or-3′)-*O*-(trinitrophenyl) adenosine 5′-monophosphate, sodium salt (TNP-AMP) was from Molecular Probes (Eugene, USA). Nucleic acid modifying enzymes and restriction

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Abbreviations: FBPase, fructose 1,6-bisphosphatase; F1,6-P_2, fructose 1,6-bisphosphate; F2,6-P_2, fructose 2,6-bisphosphate; PFK, phosphofructokinase; TNP-AMP, 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-monophosphate

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enzymes were obtained from Promega. Other reagents were from Sigma (St. Louis, USA). All the reagents were of the highest purity commercially available.

The *Escherichia coli* strain XL1-Blue MRF' Kan (Stratagene, La Jolla, USA) was used for transformation, propagation and isolation of plasmids as well as for expression of recombinant FBPases and was grown in Luria Broth at 37 °C supplemented with 100 μg/ml ampicillin [26].

Plasmid isolation, DNA restriction endonuclease analysis, ligation, and transformation were performed as described [26]. Either a Qiaprep spin miniprep kit or Qiaquick gel extraction kit (Qiagen, Hilden, Germany) was used to prepare plasmid DNA for restriction enzyme digestion, sequencing, and recovering DNA fragments from agarose gels. The sequence of each mutant gene product was confirmed by Sanger DNA sequencing on an ABI 377 sequencer using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, USA).

Mutations in the sequence of human liver and human muscle FBPases were introduced by site-directed mutagenesis using the QuikChange™ site-directed mutagenesis procedure (Stratagene). Primers used for introduction of the M177T/C179Q and T177M/Q179C mutations into the liver and muscle FBPase, respectively, were: M177/C179 5′-TGGCTCTCTCCATGGGGTGTGGCGTGGACCTC-3′ and T177/Q179 5′-CTGGTCCTTGCCACAGACCAAGGGGTCAACTGCTT-3′.

Plasmids containing single mutations E20K and K20E prepared as described previously [21] were used as template for creating triple mutants E20K/M177T/C179Q and K20E/T177M/Q179C of human liver and muscle FBPase, respectively.

Protein expression and purification were performed as described previously [21]. Protein purity and concentration throughout the purification procedure were monitored by SDS–PAGE and Bradford assays, respectively.

TNP-AMP titration of recombinant FBPases was performed according to Nelson et al. [27] using 0.5 μM concentration of FBPase. Data were analyzed by non-linear regression analysis using the following equation:

$$\Delta F/\Delta F_{\max} = L^n/K_d + L^n,$$

where ΔF is the increase of fluorescence caused by addition of ligand L ($\Delta F = F_{\text{sample}} - (\text{fluorescence of FBPase} + \text{fluorescence of TNP-AMP})$), $\Delta F_{\max}$ is the maximal increase of fluorescence caused by addition of the ligand, K_d is dissociation constant and n is the Hill coefficient.

Fluorescence data were collected using a HITACHI F4500 fluorescence spectrophotometer. All kinetic experiments were performed at pH 7.5 and 37 °C using a glucose 6-phosphate isomerase–glucose 6-phosphate dehydrogenase coupled spectrophotometric assay [28]. The reactions were started with saturating concentration of F1,6-P_2 (35 μM). One unit of enzyme activity is defined as the amount of the enzyme that catalyses the formation of 1 μmol of product per minute. Spectrophotometric measurements were performed with an Agilent 8453 diode array spectrophotometer. Determination of $I_{0.5}$, Hill coefficient for AMP and TNP-AMP as well as $A_{0.5}$ and Hill coefficient for Mg^{2+} was performed using the GraFit 4 program (Leatherbarrow, 2000). The data on the inhibition of recombinant FBPase by AMP and TNP-AMP was fitted to the following equation:

$$v/V_{\max} = [I]^n/I_{0.5}^n + [I]^n,$$

where v is the observed velocity at a specific concentration of the inhibitor, $V_{\max}$ is the velocity in the absence of the inhibitor, $[I]$ is the concentration of the inhibitor, and $I_{0.5}$ is the concentration of the inhibitor that causes 50% inhibition. The positive cooperativity the inhibitor is observed at n (Hill coefficient) > 1, whereas cooperativity is absent when $n = 1$.

All other kinetic parameters, such as k_{cat}, K_m, K_i for F2,6-P_2 and K_{is} for F1,6-P_2 were calculated with Excel and GraFit assuming an uncompetitive inhibition of the enzyme by the substrate [16,21].

3. Results and discussion

The high sensitivity of free muscle FBPase to AMP inhibition was hypothesized to be a mechanism protecting muscle cell against the loss of energy via futile cycling between PFK and FBPase [25] during muscle contraction. However, contrary to well defined mode of the inhibition of liver FBPase by AMP [27,29–31], the molecular basis of the inhibition of the muscle isozyme is unknown. Recently, we hypothesized that the many-fold higher sensitivity of muscle FBPase to AMP, when compared with the liver isozyme, is predominantly based upon the stronger binding of the inhibitor to the muscle enzyme [21]. In the running report, we demonstrate that the residues 20, 177 and 179 play a crucial role for the different binding of AMP to muscle and liver FBPase.

With the exception of the sensitivity to AMP inhibition, the kinetic properties of recombinant liver and muscle FBPases do not differ significantly (Table 1, Fig. 1). The most sensitive wild-type muscle FBPase was inhibited by AMP about 44 times stronger than the wild-type liver FBPase (Table 1, Fig. 1A). Except the residues 20, 177 and 179, all amino acids which are involved in AMP binding to the liver isozyme are also present in the muscle enzyme (Fig. 2). Thus, it might be expected that inhibition by AMP of muscle FBPase mutants in which these three amino acid residues are replaced by those found at the corresponding positions in liver FBPase should be entirely the same as the inhibition of the wild-type liver enzyme. In fact, human muscle FBPases which have been mutated towards human liver FBPase (K20E, double mutant T177M/Q179C and triple mutant K20E/T177M/Q179C) were inhibited by AMP about 22, 11 and 26 times weaker, respectively, than the muscle isozyme and only about 2–4 times stronger than the wild-type liver isozyme (Table 1, Fig. 1A).

Surprisingly, liver FBPase mutated towards the muscle isozyme (E20K, M177T/C179Q and E20K/M177T/C179Q), which had been hypothesized to be inhibited by AMP identi-

Table 1
Kinetic parameters for wild-type and mutant forms of liver and muscle FBPase

FBPase	Activity ratio: pH 9.3/7.5	k_{cat} (s^{-1})	K_m, F1,6-P_2 (μM)	K_i for F2,6-P_2 (μM)	$A_{0.5}$, Mg^{2+} (μM)	Hill coefficient (Mg^{2+})	$I_{0.5}$, AMP (μM)	Hill coefficient (AMP)
Wild-type muscle	0.23	23.4 ± 0.7	1.3 ± 0.3	0.16 ± 0.01	165 ± 16	1.89 ± 0.21	0.10 ± 0.01	1.78 ± 0.19
Muscle mutant K20E	0.34	21.1 ± 0.4	1.9 ± 0.4	0.17 ± 0.02	149 ± 26	1.78 ± 0.25	2.19 ± 0.05	1.74 ± 0.06
Muscle double mutant T177M Q179C	0.37	19.9 ± 0.8	1.5 ± 0.5	0.13 ± 0.04	203 ± 34	1.82 ± 0.34	1.14 ± 0.12	1.74 ± 0.23
Muscle triple mutant K20E T177M Q179C	0.31	17.6 ± 0.6	1.7 ± 0.4	0.16 ± 0.03	179 ± 23	1.98 ± 0.31	2.62 ± 0.13	1.83 ± 0.12
Wild-type liver	0.34	20.8 ± 0.5	2.1 ± 0.3	0.23 ± 0.04	199 ± 32	1.83 ± 0.19	4.41 ± 0.10	2.08 ± 0.08
Liver mutant E20K	0.38	18.6 ± 0.8	2.3 ± 0.5	0.24 ± 0.05	219 ± 41	1.89 ± 0.23	4.82 ± 0.14	2.05 ± 0.10
Liver double mutant M177T C179Q	0.42	18.4 ± 0.5	1.9 ± 0.6	0.19 ± 0.04	241 ± 53	1.78 ± 0.19	4.22 ± 0.14	2.03 ± 0.1
Liver triple mutant E20K M177T C179Q	0.26	21.7 ± 0.3	1.5 ± 0.2	0.18 ± 0.03	187 ± 29	1.91 ± 0.27	3.15 ± 0.15	1.75 ± 0.11

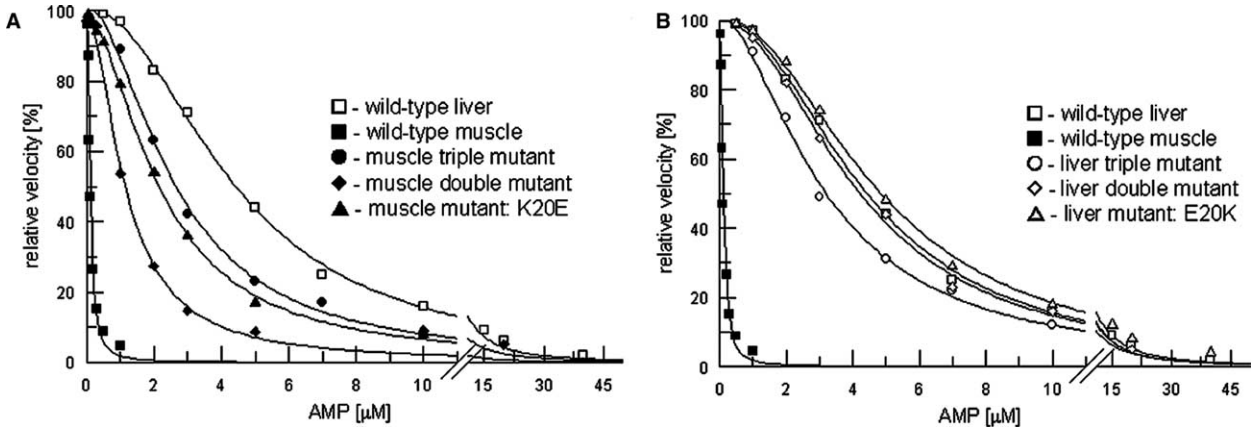

Fig. 1. The inhibition of recombinant FBPases by AMP. Hill coefficients were close to 2.0 for all recombinant FBPases.

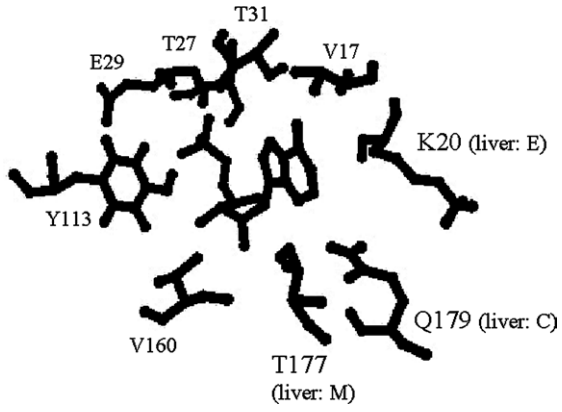

Fig. 2. Human muscle FBPase AMP binding pocket. AMP is located in the middle of the picture at a position in which it is bound to the human liver enzyme. The differences between muscle and liver FBPase are marked in bold. The structure of human muscle FBPase was constructed on the basis of 1FTA.pdb using SPDBV software [32–34].

cally to wild-type muscle FBPase, was inhibited by AMP similarly to the wild-type liver enzyme (Table 1, Fig. 1B). This result is in contradiction with the data obtained for the muscle mutants showing the key role of residues 20, 177 and 179 in the high sensitivity of muscle FBPase to AMP.

In order to distinguish whether the differences in the inhibition between wild-type and mutated FBPases were due to a different binding or to a distinct mechanism of allosteric inhibition, the fluorescent AMP analogue, TNP-AMP [27], was used. TNP-AMP inhibited FBPases similarly to AMP (Table 1, Fig. 3A and B), and the only significant difference was the absence of cooperativity in the binding of and in the inhibition by the AMP analogue (Figs. 3 and 4A and B) [27]. For the muscle FBPase K20E and triple mutants, dissociation constants for TNP-AMP were comparable with that determined for the wild-type liver enzyme (Fig. 4A). This suggests that the low sensitivity of the muscle triple mutant to AMP inhibition predominantly originates from the low affinity of the mutant to AMP, which is caused by the substitution of K20 with E and/or the substitutions of T177 with M and Q179 with C. Since the binding of TNP-AMP to muscle double mutant was much stronger (K_d was 0.84 μM, Fig. 4A) than the binding of TNP-AMP to the muscle single and triple mutants, thus, it may be presumed that K20 plays the key role in the high affinity of the muscle enzyme to AMP.

In the case of the triple and the double mutant of liver FBPase, the dissociation constants for TNP-AMP were, respectively, comparable with that determined for the wild-type muscle enzyme and about two times lower than that determined for the wild-type liver enzyme (Fig. 4B). On the

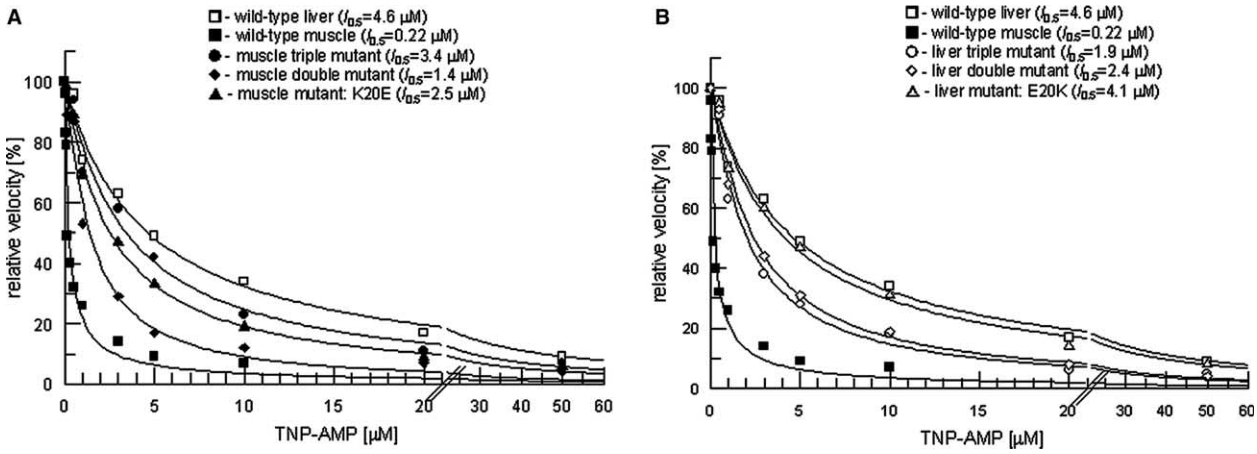

Fig. 3. Inhibition of recombinant FBPases by TNP-AMP. The concentrations of the inhibitor which causes 50% inhibition of the enzyme activity ($I_{0.5}$) are presented in bracelets. Hill coefficients were close to unity for all recombinant FBPases.

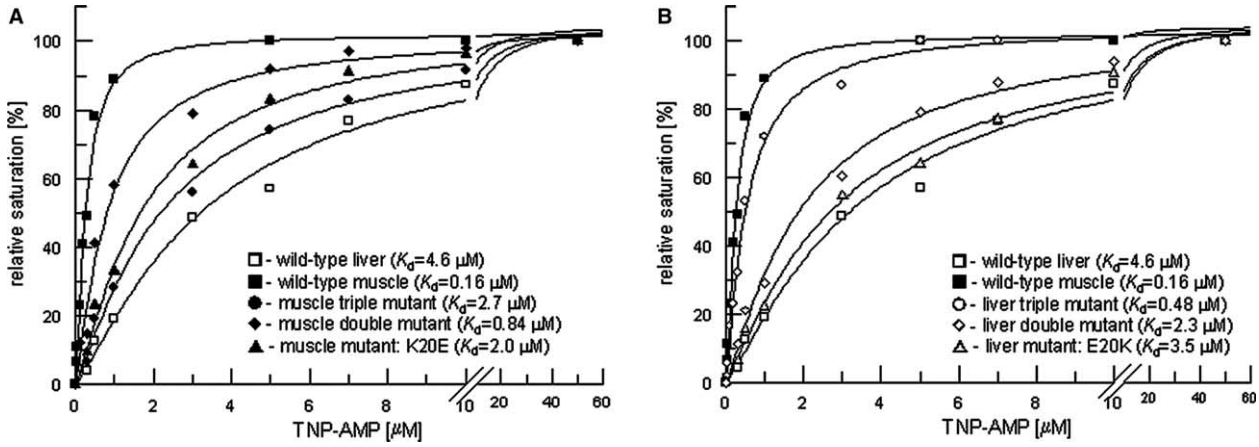

Fig. 4. TNP-AMP titration of the recombinant FBPases. Fluorescence of TNP-AMP was observed in the presence of 0.5 μM FBPase. A wavelength of 410 nm was used for excitation and fluorescence emission was monitored at 535 nm. Dissociation constants (K_d) are presented in bracelets. Hill coefficients were close to unity for all recombinant FBPases.

other hand, the inhibition of the liver mutants by TNP-AMP (Fig. 3B) as well as by AMP (Fig. 1B) was relatively weak and essentially the same as that of the liver isozyme.

If the mechanism of the molecular changes leading to the inhibition were not impaired by mutations, the stronger binding of the inhibitor to the liver mutants should result in the stronger inhibition. The observed discrepancy between the strength of the inhibitor binding and the level of half-inhibition, which is especially evident in the case of the liver triple mutant, suggests that the binding of AMP to the muscle-like AMP cavity within liver-type domain somehow disturbs the way of the inhibitory signal transmission.

It has been proposed that AMP inhibits liver FBPase stabilizing an inactive state of mobile loop 52–72 of the adjacent monomer by its interaction with residues 187–194 and the N-terminal region [27,30]. Thus, it may be hypothesized that the association of AMP to the residues T177, Q179 and K20 which are in close proximity to the regions involved in the stabilization of an inactive form of wild-type liver FBPase, impairs their interaction with loop 52–72 and hence, makes the inhibition weaker.

On the other hand, the observed discrepancy also suggests that an additional mechanism connected with the different pathway of allosteric signal transmission in muscle FBPase should be taken into account to explain the strong inhibition of the muscle isozyme by AMP. An attractive hypothesis is that the presence of highly conserved N-terminal residues in muscle FBPase might be responsible for the stronger stabilization of an inactive form of the muscle isozyme [21,30].

The main difference between the liver E20K mutant and wild-type muscle FBPase is the presence of M177 and C179 in the mutant protein, and it seems obvious that these two residues are solely responsible for the decreased affinity of the mutant enzyme towards the inhibitor (Fig. 4B). Therefore, although lysine 20 is presumed to be crucial for the strong binding of AMP to the muscle enzyme, it seems that its presence is not sufficient to overcome the destabilizing effect exerted by residues 177 and 179 on the binding of AMP to liver FBPase (Fig. 4B).

In summary, the results presented here strongly support the hypothesis that the presence of K20 and the lack of amino acids which destabilize the interaction between AMP and FBPase (M177 and C179) is crucial for the much stronger binding of the inhibitor to muscle FBPase than to the liver isozyme. However, the strong inhibition of the muscle isozyme does not exclusively arise from the stronger binding of AMP but also from another mechanism of intramolecular changes leading to the inhibition.

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